Unlocking microalgal treasures

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Summary

1. Summary

Currently, Indonesia is known as the largest CPO producer in the world, followed by Malaysia and Thailand. During the production, about 1 ton of fresh fruit bunch (FFB) produces 0.66 ton palm oil mill effluent (POME). The pH of raw POME ranges between 4.0 and 5.0, and its chemical oxygen demand (COD) and biological oxygen demand (BOD) are extremely high. Furthermore, POME contains a high amount of total suspended solids (TSS), total dissolved solids (TDS) and volatile suspended solids (VSS). It is therefore evident that comprehensive treatment should be done to meet standard regulations before POME can be released into the environment (i.e. rivers, lakes). To treat POME, several mechanical, chemical or biological methods have been developed. However, compared to other techniques, biological treatment is more environmental friendly, less expensive as well as energy saving. Over the past decades, the utilization of agricultural waste(water) with the aim to meet industrial demands and to generate useful products has gained increasing interest. Valorization in this context is the process of converting waste materials into valuable products by using microorganism such as yeast, bacteria, and microalgae. In general, the utilization of microalgae to treat wastewater is more preferable since the substances produced by these organisms may have a high economic value. At the start of this PhD project, the utilization of POME by microalgae was so far mainly focused on lipid production, bulk biomass and wastewater treatment: the production of high value-added compounds with an eye towards large scale cultivation was hardly considered. Therefore, the goal of this thesis was to fill the gaps in this knowledge by: i) investigating the potential of growing several microalgal species on POME and to monitor the production of their associated high value products, ii) investigating a range of environmental and nutritional parameters and their interrelation, to optimize the utilization of POME as growth medium for key microalgae and their associated value added products, iii) investigating the potential of microalgal cultivation to improve POME quality through color and phenolic compound removal. These research aims were translated into specific goals, and subsequently addressed in four research chapters.

In **chapter 2**, we explored the utilization of POME by the marine diatom *Phaeodactylum tricornutum* and investigated a variety of environmental conditions in order to optimize fucoxanthin productivity at different irradiance, temperature and nutrient conditions. Fucoxanthin produced by diatoms such as *P. tricornutum*

can exhibit anti-cancer, anti-obesity, and anti-diabetic activity. I hypothesized that the fucoxanthin productivity of *P. triconutum* grown on POME depended on (a combination of) irradiance level, nutritional ratios, salinity and temperature. First, optimum irradiance conditions for fucoxanthin productivity were determined for *P. tricornutum* growing on artificial medium. This optimum irradiance was applied in all subsequent experiments involving POME fractions. Then, the impacts of various POME fractions on growth rate and fucoxanthin productivity were investigated and selected nutrient additions were done to optimize fucoxanthin productivity. Box-Behnken design (BBD) response surface methodology (RSM) was employed to reveal the optimum combination of environmental conditions and to understand the interaction of salinity, temperature and nutrient concentration with respect to fucoxanthin productivity. Through this stepwise experiments, we found that *P. tricornutum* may be used for large scale cultivation on 30% v/v POME with the aim to produce fucoxanthin. BBD RSM revealed that optimum fucoxanthin productivity was influenced by temperature, salinity and the addition of urea. Nutrient enrichment by phosphorus did not enhance cell density and fucoxanthin productivity, while urea addition was found to stimulate both. Furthermore, optimal urea (85 mg L^{-1}), temperature (23 °C) and salinity (22-23 PSU) conditions were determined from our final experimental series.

Since recently, the interest in utilizing exopolysaccharide (EPS) from microalgae has increased. In **chapter 3**, the production of sulfated exopolysaccharide (sEPS), a specified EPS byproduct synthesized by *P. tricornutum*, was studied by using POME as growth medium. In the pharmaceutical field, sEPS from *P. tricornutum* is promising as anti-inflammatory, antiviral, antiparasitic, anti-tumor, and hypocholesterolemic substance. However, the production of sEPS at a large scale is limited due to the high production costs. Alternatively, the cultivation of microalgae on POME with the aim to produce sEPS could be promising, given the relatively high nutrient content of the wastewater, thereby making commercial nutrients partly or completely redundant. Furthermore, the treated, sEPS containing POME might be utilized for soil improvement in the vicinity of the palm oil plants. Microalgae tend to produce higher sEPS under stressed conditions such as excess irradiance and supra-optimal temperatures, as a means to prevent cell damage via the action of exopolymers. Other factors such as nutritional conditions and salinity were also reported to influence EPS production and composition. It was therefore hypothesized that the production of sEPS by *P. tricornutum* grown on POME would be influenced by environmental and nutritional conditions. To confirm this, a series of four step-wise experiments was done by: I: determining the optimal POME concentration for *P. tricornutum* growth, II:studying the effect of nutrient additions on *P. tricornutum* biomass and sEPS production when growing on 30% POME, III: applying BBD RSM to unravel the optimal conditions for temperature, salinity, and urea addition, and IV: studying the effect of nutrient enrichment on nutrient removal efficiency of POME. The results showed that *P. tricornutum* is a suitable candidate for effective cultivation on 30% POME while producing sEPS at the large scale, when salinity requirements are met at 20-35 PSU. By using BBD RSM, we found that the interaction of high urea (100 mg L^{-1}) and relatively high temperature (25°C) stimulated both growth rate and sEPS production. Furthermore, the addition of urea to the POME medium was found to stimulate phosphorus removal from POME by *P. tricornutum*.

In **chapter 4**, the brackish cyanobacterium *Arthrospira platensis* was cultivated on high POME fractions after which the production of the value-added product, the pigment C-phycocyanin, was explored as a function of different cultivation modes as well as environmental and nutritional conditions. As reported earlier, a high POME fraction can be utilized by *A. platensis* when grown in continuous cultivation mode. However, continuous cultivation is not easily applicable in large scale systems. For the present study it was therefore hypothesized that semi-continuous cultivation could enhance the biomass and C-PC productivity of *A. platensis* cultured on POME medium after optimizing nutrient and other environmental conditions. To confirm this hypothesis, a stepwise approach was followed: first we investigated the interactive effect of irradiance and nitrogen concentration on *A. platensis* biomass and C-PC productivity, grown on standard growth medium. Secondly, growth and C-PC productivity of *A. platensis* were studied on different POME fractions. Then, environmental and nutritional conditions were further investigated to determine the interactive effects of light intensity, urea, salinity, and POME concentration on C-PC productivity. The optimum urea concentration as promising nitrogen source was investigated given the possible toxic effects at higher urea concentrations. Furthermore, salinity was optimized since POME contains a relatively low salinity. Finally, *A. platensis* was cultured in a semi-continuous mode at varying nutrient conditions by adding urea or phosphorus, in order to unravel the impact of N:P ratio on biomass and C-PC production during semi-continuous cultivation. In this study, we found that irradiance and nitrogen concentration were the main factors driving C-PC productivity. Based on central composite design (CCD) RSM, the optimal salinity was found to be 22.5 PSU, and no inhibition was found up to 813 mg L^{-1} of urea when employing batch cultivation. By applying semi continuous cultivation with 50% POME at the first stage and 100% POME at the second stage, C-PC productivity was higher compared to batch cultivation with the addition of urea, and could reach productivity levels as high as the artificial control Zarrouk medium during batch cultivation.

In **chapter 5**, we investigated the removal of phenolic compounds and color on POME by using *A. platensis*. To the best of our knowledge, information on color and phenolic compound removal from POME using microalgae was so far lacking.

In the present study, factors affecting phenolic compound and POME color removal were considered by including photodegradation and microalgal activity by varying the initial phenol concentration in POME, irradiance, POME fractions, external nitrogen addition, and salinity. A range of control experiments (without microalgae) was done, to unravel the contribution of photodegradation to the removal processes under consideration. In this study, we found that POME fractions influenced growth rate, final biomass, absolute COD removal, and absolute color removal by the activity of *A. platensis*. Based on factorial design, salinity, nitrogen addition, and initial POME concentration did not influence total color removal. Based on CCD RSM, the addition of phenolic compounds as gallic acid in POME at high light intensity could increase the growth rate up to 0.45 d^{-1} and final biomass up to 400 g L^{-1} while on the other hand total phenolic compounds were removed almost completely (94%). Photodegradation activity contributed significantly to phenolic compound removal. However, *A. platensis* activity was higher compared to photodegradation when removing phenolic compounds at low light condition. High phenolic compound removal can thus be achieved by the combination of *A. platensis* activity and photodegration.

2. Implications and recommendations

Palm oil plays a primary role in the oil crops worldwide. It is still the most economical source of vegetable oil due to its high productivity (volume/ area/ time) and its relatively low requirement of productive land to grow compared to other crops. This industry has a huge contribution on the revenue in Indonesia as well as other countries in the region of South East Asia. However, the palm oil industry faces serious issues with respect to sustainability, including the many negative impacts to the environment.

Since 2014, more strict regulatory standards have been set for POME quality before it can be discharged into the environment. However, the POME discharge by medium to small factories, which commonly use cheap and conventional ponding systems to treat the wastewater, does not meet the regulatory standard due to the ineffectiveness of the treatment technology. Some factories mix POME with empty fruit bunch (EFB) for compost production. However, the high ratio of EFB to POME, long composting period, and land requirements to process and store it, are still not answering the problems. Other options are also available such as installing membrane filtration, evaporating POME to minimise discharge of the wastewater effluent, and digesting POME using bioreactor digesters. Among other management wastewater treatments, installing bioreactor digesters seems promising to make the industry more sustainable, since not only the pollutants can be lowered, but also the biogas produced from this system can be utilized by the factory itself.

Generally, microalgae are cultivated on wastewater to obtain carbohydrates, lipids, and proteins which are mainly focused on fuel and animal feed. However, microalgae are known to produce high value bioactive compounds, which may have different pharmaceutical and cosmetic applications. In this thesis, we have successfully grown the marine diatom *Phaeodactylum tricornutum* on POME during which the brown pigment fucoxanthin, and the byproduct sulphated exopolysaccharide, were produced. C-Phycocyanin, an antioxidant produced by *A. platensis*, was also enhanced by applying semi-continuous cultivation mode on high POME fractions. By utilizing POME as growth medium for microalgae, the cost of the bioactive compounds produced by microalgae might be reduced, since synthetic media can be replaced by suitable POME fractions. Furthermore, the treated, sEPS containing POME might be utilized for soil improvement in palm tree plantations.

Despite its high nutrient levels, the utilization of POME as growth medium for microalgae at industrial scale is still challenging. First, the high levels of organic compounds, consisting of tannins, lignin, and phenolic compounds could negatively affect growth. The dark coloration due to the high concentrations of suspended solids could inhibit light penetration, which is a critical factor for photosynthetic growth. Moreover, the presence of heterotrophic bacteria may affect biomass productivity. The pH and salinity of the wastewater needs to be adjusted before it can be used as growth medium for alkaline microalgae such as *A. platensis* or marine microalgae, such as *Phaeodactylum tricornutum*. Heavy metals contained in POME might be lowering the quality of the microalgal product, and might be harmful for food and pharmaceutical applications.

However, the conditions as mentioned above could be prevented by employing some pretreatment process to lower COD, color, and heavy metals from the POME as described previously. To increase the salinity, cultivation might be relocated to seashore areas, to allow for the utilization of natural seawater. Furthermore, the wastewater could be blended with hypersaline wastewater generated from industrial activities, such as chemical manufacturing and oil production, to make the cultivation become more feasible for marine microalgae. Finally, it should be considered that the type of cultivation system such as closed cultivation using photo-bioreactors versus open (pond) cultivation could also influence the growth, biomass, and the value-added products. It may be clear that more research must be done to optimize POME utilization for high-value product generation by microalgae. The optimization should be done based on a combination of approaches such as appropriate pretreatments, choice of species, cultivation modes, cultivation conditions, nutrient ratio's, before the valorization of POME becomes economically feasible.

3. Concluding remarks (Future outlook)

It is predicted that bioenergy and high-value biochemical production from microalgae cultivated on palm oil mill effluent become more feasible in the future if the owners implement a wastewater treatment system based on bioreactor digesters. Microalgae can be more easily cultured on the digested POME which contains lower COD and toxic substances. The cultivation can be integrated with the biogas purification system where CO_2 can be utilized for photo-autotrophic cultivation. By integrating this, a higher heating value biogas can be obtained, and high value-added pigments from microalgae can be obtained at a lower price compared to common cultivation based on commercial fertilizers. Some modifications such as nitrogen limitation and light saturation can be used to enhance some carotenoid pigments such as beta carotene and astaxanthin.

A biorefinery concept could also be applied in the future, by applying specified separation techniques to produce derivative products from microalgae. For example, a single cell of microalgae could produce lipid, protein, carbohydrate, and pigments, which can be converted to fuel, feed, polymers (bioplastics, nano carbon composite), and antioxidants (fucoxanthin, phycocyanin, astaxanthin). By implementing this concept, the production cost could be further minimized, thus lowering the price of the products.